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THE ROLE OF NAD^+ DEPLETION IN THE MECHANISM OF SULFUR MUSTARD-INDUCED METABOLIC INJURY

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Results of our previous studies on the chemical warfare agent sulfur mustard (2,2'-dichlorodiethyl sulfide) suggested that mustard-induced inhibition of glycolysis is not solely a function of NAD^+ depletion. To define the role of NAD^+ in mustard-induced metabolic injury, we examined the effects of mustard \pm niacinamide on energy metabolism in cultured human keratinocytes. Sulfur mustard caused concentration-dependent decreases in viable cell number and ATP content at 24 hours, but not earlier, and time- and concentration-dependent glycolytic inhibition and NAD^+ depletion as early as 4 hours. Niacinamide partially protected NAD^+ levels at all time points, but did not prevent adverse effects on glycolysis, intracellular ATP, or viable cell number. These results support our earlier conclusions and suggest that sulfur mustard may inhibit glycolysis directly.

Keywords: Cell culture; Glycolysis; HD; Keratinocytes; NAD^+ ; Niacinamide; Sulfur mustard; Toxicology
Abbreviations: SM (sulfur mustard, 2,2'-dichlorodiethyl sulfide); HEK (human epidermal keratinocytes); PARP (poly (ADP-ribose) polymerase)

INTRODUCTION

Sulfur mustard (2,2'-dichlorodiethyl sulfide, SM) (1) is a vesicating (i.e., blister-forming) chemical warfare agent for which there is currently no antidote. It is also a potent alkylating agent, inducing cross-links and strand breaks in DNA, as well as covalent modification of proteins and other cellular components. As a result, its effects on cells and tissues are varied and complex, and despite years

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of research, the mechanism(s) by which SM induces blister formation is not understood.

One of the prevailing theories of SM-induced vesication (2) involves the enzyme poly(ADP-ribose) polymerase (PARP). According to this hypothesis, PARP is activated by SM-induced DNA damage; activation of PARP then leads to rapid depletion of intracellular NAD^+ , inhibition of cellular energy metabolism, protease release, and cell death. To date, a number of components of the PARP hypothesis have been demonstrated experimentally, such as SM-induced PARP activation, NAD^+ depletion, metabolic inhibition and protease release (1). In addition, niacinamide, a PARP inhibitor and precursor for NAD^+ synthesis has been reported to provide at least partial protection against cytotoxic, histopathologic and NAD^+ -depleting effects of SM in cultured cells (3–9), human skin explants (10), and *in vivo* in the hairless guinea pig (11).

In our laboratory, we have demonstrated (12) that exposure of cultured human epidermal keratinocytes (HEK) to SM leads to time- and concentration-dependent cytotoxicity, NAD^+ depletion and inhibition of glucose metabolism. However, we observed that the inhibition of glycolysis induced by SM was only partially correlated with the depletion of NAD^+ and, thus, was not simply a function of changes in the content of NAD^+ . Rather, SM appeared to induce complex shifts in the pattern of glucose metabolism that depended on the severity of the exposure.

To better define the roles of NAD^+ and niacinamide in the mechanism of SM-induced metabolic injury and its prevention/reversal, we have carried out a systematic study of the effects of SM and niacinamide on several aspects of energy metabolism in cultured HEK. The results are presented herein.

MATERIALS AND METHODS

Materials

Human epidermal keratinocytes, Keratinocyte Growth Medium (KGM), Trypsin-EDTA, and Trypsin Neutralizing Solution were purchased from Cambrex (Walkersville, MD). Sulfur mustard was obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). Stock SM (4 mM) was suspended in KGM; all further dilutions were made using KGM. Enzymes and fine chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other chemicals were reagent grade or better and purchased from commercial suppliers.

SM Exposure and Niacinamide Treatment

HEK purchased as 2nd passage cells were maintained in KGM at 37°C under 5% CO_2 and subcultured as previously described (3). Third to 5th passage cells were plated at 50–100,000 HEK/well in 24-well Falcon tissue culture plates (Becton-Dickinson & Co., Lincoln Park, NJ) in 1 mL of KGM. Upon reaching approximately 80% confluence, the KGM was replaced with fresh KGM supplemented with 0, 0.01, 0.1, or 1.0 mM niacinamide. SM in KGM was added immediately after niacinamide to a final concentration of 0, 100, or 500 μM . Each condition

was carried out in duplicate wells. The plates were allowed to stand 1 h at room temperature in a fume hood for venting of SM, after which they were returned to the incubator.

Cell counts and viabilities were determined for HEK harvested from both normal and exposed/treated wells with trypsin-EDTA (3). Cells were counted manually using a hemacytometer or in a Coulter Counter ZM (Coulter Electronics, Hialeah, FL). Viabilities were determined by dye exclusion methods, measuring uptake of either trypan blue in a hemacytometer or propidium iodide in a Coulter EPICS C flow cytometer (3). In each case the choice of technique was solely based on instrument availability. Neither the cell counts nor the viabilities were significantly affected by the measurement technique used.

Extractions

Cells plus medium were acid extracted with 0.5 M HClO_4 immediately upon addition of the fresh medium ($t = 0$ h) and at timed intervals up to 24 h. The samples were allowed to extract overnight at 4° and neutralized with 2 N KOH that had been prepared in 0.66 M phosphate buffer, pH 7.5 (13). Precipitated KClO_4 was removed by centrifugation and the supernatants stored frozen until assay. In experiments for which ATP assays were needed, the cells plus medium were not acid extracted, but rather were solubilized with detergent (Somatic Cell ATP Releasing Reagent, Sigma-Aldrich, St. Louis, MO) prior to storage at -70° . The latter extraction method increases the sensitivity of the ATP assay by minimizing salt-induced quenching effects. It did not alter the results of NAD^+ or glucose/lactate assays.

In some experiments, plates were centrifuged to separate cells from medium prior to extraction. The plates were centrifuged at room temperature for 10 min at $250 \times g$ in a Beckman Model GS-6R tabletop centrifuge (Beckman Instruments, Fullerton, CA) fitted with a Beckman Model GH-3.7 swinging bucket rotor and microtiter plate carriers. The medium was aspirated and saved. Both the supernatant and pelleted cells were then extracted as described earlier.

Assays

The NAD^+ contents of the extracted HEK were assayed in a final volume of 1.1 mL using the enzymatic cycling assay of Jacobson and Jacobson (13) modified as previously described (12). The coenzyme contents were calculated from standard curves constructed using NAD^+ solutions of known concentration determined by enzymatic endpoint assays (14).

Glucose and lactate levels in the extracts were assayed directly using a YSI Model 2700 dual analyzer (Yellow Springs Instruments, Yellow Springs, OH) fitted with glucose- and lactate-selective enzyme membranes. The rates of glucose metabolism were measured as the disappearance of glucose and the appearance of lactate with time, and were calculated using linear least squares regression analysis.

ATP was assayed using the Sigma ATP bioluminescent assay kit according to the instructions provided (Sigma Technical Bulletin #BSCA-1, Sigma-Aldrich, St. Louis, MO, 1987). Assays were carried out in a 96-well "Removawell" microtiter

plate system fitted with 1 mL white, opaque inserts (Dynatech Laboratories, Inc., Chantilly, VA). Bioluminescence was measured in a Cytofluor Series 4000 fluorescence plate reader using a 580 nm/50 nm bandwidth emission filter (PerSeptive BioSystems, Framingham, MA). The signal was calibrated using ATP standards provided in the assay kit.

Statistical Analysis

The data were analyzed for statistical significance using 2-Way Analysis of Variance (ANOVA) with SM and niacinamide as factors. When significant differences were revealed in the ANOVA, differences were isolated using All Pairwise Multiple Comparison Procedures (Tukey Test). $p < .05$ was considered significant.

RESULTS

Cell Counts and Viabilities

The cell viabilities, measured as the ability of HEK to exclude trypan blue or propidium iodide at 24 h after exposure to SM and/or niacinamide (given as percentage of the total cell population), are shown in Fig. 1. At 100 μ M, SM had no effect, whereas 500 μ M SM caused an approximately 30% loss in viability of the cells present in each sample. Cell viability was not affected by SM at 8 h or less after exposure (not shown). Niacinamide did not protect against the viability loss in these experiments.

The effects of SM and niacinamide on the number of cells in each well at 24 h after exposure are shown in Fig. 2. The total cell counts include both "viable" and "non-viable" cells as determined by dye exclusion. No significant loss of cells was

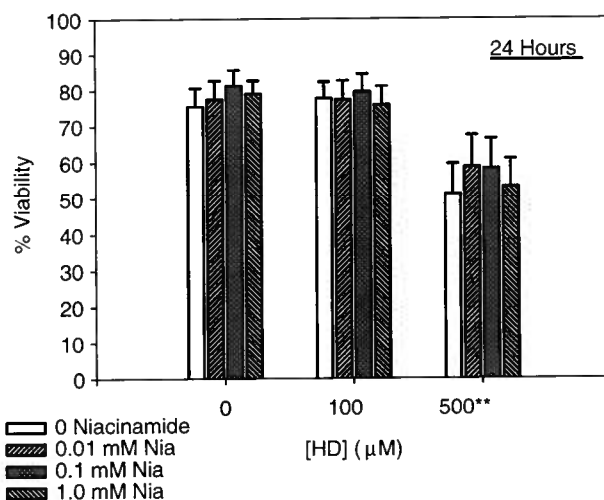


Figure 1 Effects of SM and niacinamide on 24-hour viability of HEK as measured by dye exclusion. Experimental conditions were as described in Materials and Methods. Data are expressed as Mean \pm SEM ($n = 11$). **Significantly different from 0 and 100 μ M SM.

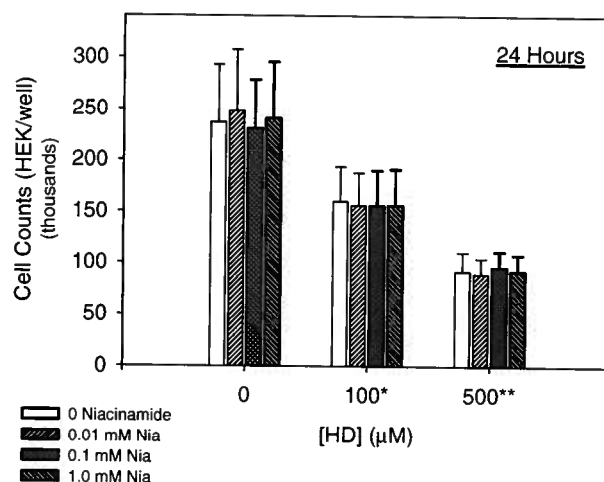


Figure 2 Effects of SM and niacinamide on 24-hour cell counts of HEK. Experimental conditions were as described in Materials and Methods. Data are expressed as Mean \pm SEM ($n = 8$). *Significantly different from 0 SM; **Significantly different from 0 and 100 μ M SM.

seen at 4 or 8 h after exposure to SM (not shown). However, at 24 h both 100 and 500 μ M SM significantly decreased the total cell counts at this time point to 66% and 38% of untreated controls (0 SM; 0 niacinamide), respectively. Niacinamide did not protect against this cell loss.

The decrease in cell numbers observed at 24 h after SM exposure results, most likely, from a combination of decreases in cell proliferation and necrotic cell loss. When combined with the loss in viability of the remaining cell population, 500 μ M SM decreased the number of viable cells even further to only 27% of untreated controls. In this study, the results of the biochemical assays were normalized to the viable cell count rather than the absolute cell count.

Glucose Metabolism

The effects of SM and niacinamide on the rates of glucose metabolism by cultured HEK are shown in Fig. 3. Both 100 and 500 μ M SM inhibited glucose utilization more severely than lactate production. Glucose utilization was inhibited by 43% at 100 μ M SM and 73% at 500 μ M SM, whereas these same concentrations inhibited lactate production by a lesser amount (27% and 62%, respectively). Addition of 0.01, 0.1 (not shown) or 1 mM niacinamide (Fig. 3) provided no significant protection against SM-induced inhibition of glucose metabolism in these experiments.

NAD⁺ Content

Figure 4 shows the effects of SM and niacinamide on the NAD⁺ levels of HEK in culture at 4, 8, and 24 h after exposure. At all three time points, 500 μ M SM caused significant decreases in intracellular NAD⁺, the extent of which increased as

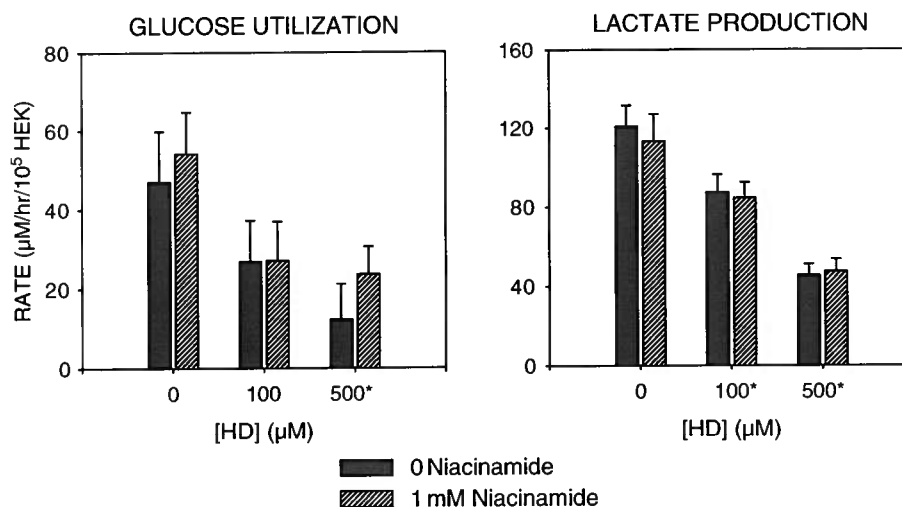


Figure 3 Effects of SM and niacinamide on glucose utilization and lactate production by HEK. Experimental conditions were as described in Materials and Methods. Data are expressed as Mean \pm SEM ($n = 12$). *Significantly different from 0 SM; **Significantly different from 0 and 100 μM SM.

time progressed from 30% depletion at 4 h to 45% at 8 h and 55% at 24 h after exposure. At 100 μM SM, NAD^+ depletion was only seen at 24 h, and even then the level was only decreased by 20% compared with untreated controls.

At all three time points, inclusion of niacinamide in the medium at a concentration of 1 mM significantly increased intracellular NAD^+ in normal and SM-exposed HEK to between 140% and 160% of the values measured either in the absence of niacinamide or at lower concentrations. For our purposes, we consider protection to be the ability of a treatment to maintain NAD^+ levels at or near those of untreated controls. By this criterion, 1 mM niacinamide provided at least partial protection against SM-induced NAD^+ depletion in all cases. The extent of this protection depended on both the concentration of SM used and the time after exposure at which samples were taken for assay. The NAD^+ depletion caused by 100 μM SM was completely prevented at 24 h, whereas at 500 μM SM the degree of protection diminished with time. In the latter case, NAD^+ levels were 95%, 78%, and 58% of untreated controls at 4, 8, and 24 h, respectively.

Because 500 μM SM caused a dramatic decrease in the number of viable cells remaining in the cultures at 24 h after exposure, we corrected the data in Fig. 4 for the actual cell count at each data point. The results are presented in Fig. 5, from which one can see that while SM had no apparent effect on the levels of NAD^+ per viable cell, 1 mM niacinamide retained its ability to increase NAD^+ levels.

In another set of experiments, the results of which are presented in Table 1, we found that upon centrifugation of cells that had been incubated for 24 h with 500 μM SM with or without niacinamide, 50%–60% of the total NAD^+ measured was contained in the supernatant. This was not seen with 100 μM SM; at this concentration the proportion of NAD^+ appearing in the supernatant did not increase over that of untreated controls. In no case did niacinamide alter the results.

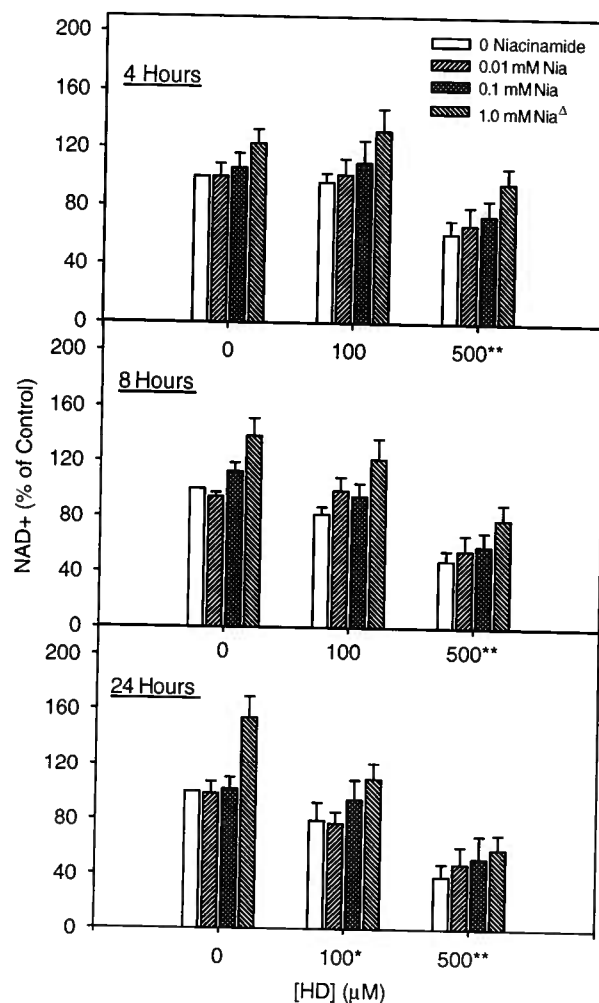


Figure 4 Effects of SM and niacinamide on the NAD⁺ contents of HEK. Experimental conditions were as described in Materials and Methods. Data are expressed as Mean \pm SEM ($n = 14, 10$, and 13 for $4, 8$, and 24 h, respectively). Control values (0 SM; 0 Nia): 255 ± 58 pmoles/ 10^5 HEK (4 hr); 215 ± 62 pmoles/ 10^5 HEK (8 hr); 402 ± 105 pmoles/ 10^5 HEK (24 hr). *Significantly different from 0 SM; **Significantly different from 0 and $100 \mu\text{M}$ SM. ^ΔSignificantly different from 0 Niacinamide.

ATP Content

As can be seen in Fig. 6, exposure of HEK to $100 \mu\text{M}$ or $500 \mu\text{M}$ SM did not significantly deplete the intracellular stores of ATP until 24 h after exposure. At this time point the ATP depletion was concentration-dependent, being reduced to 75% of untreated controls by $100 \mu\text{M}$ and to only 6% of untreated controls by $500 \mu\text{M}$ SM. No effect of SM was seen at 8 h, whereas at 4 h after exposure $100 \mu\text{M}$ SM increased the levels of ATP by 45% .

Niacinamide did not protect against the loss of ATP seen at 24 h after exposure. At 8 h, all concentrations of niacinamide decreased the ATP levels by 25% – 40% in

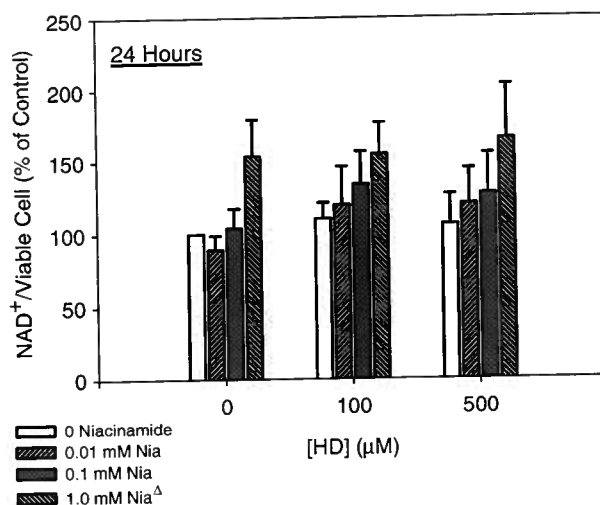


Figure 5 NAD⁺ contents of HEK corrected for SM-induced cell loss. Data are expressed as Mean \pm SEM (n = 8). ^ΔSignificantly different from 0 and 0.01 mM Niacinamide.

Table 1 Effect of HD on release of NAD⁺ from HEK at 24 hours after exposure

	% of NAD ⁺ in Supernatant		
	0 HD	100 μM HD	500 μM HD
0 Niacinamide	26.9 \pm 0.5	15.5 \pm 3.0	53.5 \pm 4.9**
0.01 mM Niacinamide	28.4 \pm 5.7	23.4 \pm 9.4	53.7 \pm 8.7**
0.1 mM Niacinamide	21.6 \pm 4.3	12.4 \pm 2.1	61.6 \pm 5.2**
1.0 mM Niacinamide	22.2 \pm 1.2	16.4 \pm 2.2	55.6 \pm 10.3**

Experimental conditions are as described in Materials and Methods. Data are expressed as Mean \pm SEM (n = 2). **Significantly different from 0 and 100 μM SM.

both the presence and absence of SM. However, this decrease may not be physiologically significant because niacinamide alone did not cause a loss of viable cells (see Figs. 1 and 2). Niacinamide had no effect on ATP at 4 h after exposure.

When the 24-h data were corrected for the effect of SM on cell number (Fig. 7), the ATP contents of the cells exposed to 100 μM SM were found to be comparable to those of untreated controls. At 500 μM SM, however, ATP remained severely depleted and was not protected by niacinamide.

DISCUSSION

The effects of SM exposure on energy metabolism in cultured HEK reported herein are in agreement with our earlier results (12) as well as with those reported by other investigators (1, 3, 4, 7-9). That is, we have observed that SM causes time-

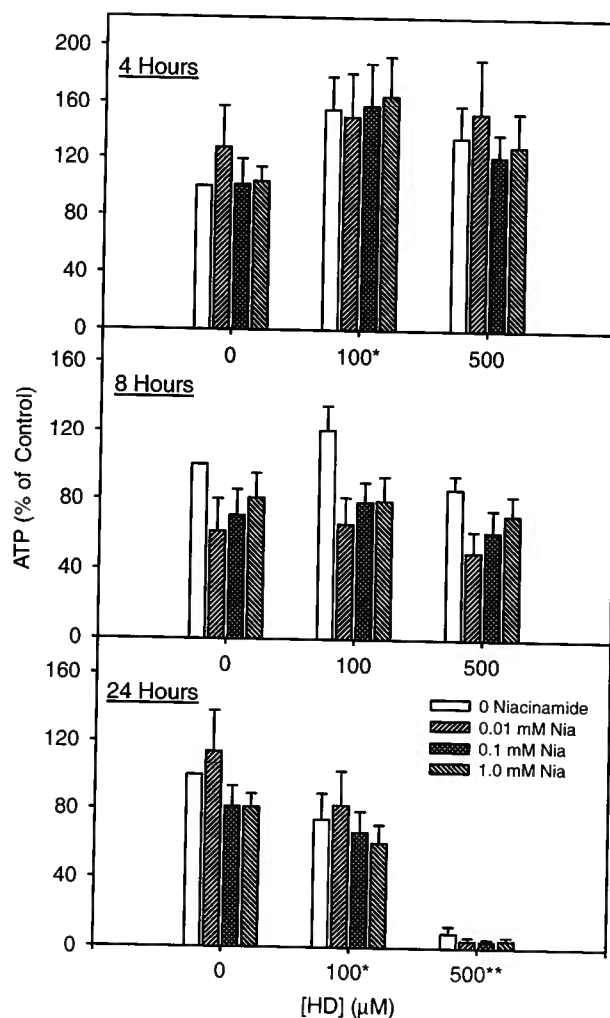


Figure 6 Effects of SM and niacinamide on the ATP contents of HEK. Experimental conditions were as described in Materials and Methods. Data are expressed as Mean \pm SEM ($n = 7, 6$, and 7 for $4, 8$, and 24 hours, respectively). Control values (0 SM; 0 Nia): 1.0 ± 0.3 pmoles/ 10^5 HEK (4 hr); 1.9 ± 0.2 pmoles/ 10^5 HEK (24 hr). *Significantly different from 0 SM; **Significantly different from 0 and $100 \mu\text{M}$ SM.

and concentration-dependent cytotoxicity, NAD^+ and ATP depletion as well as inhibition of glycolysis. We further conclude from our data that $500 \mu\text{M}$ SM causes (a) a significant loss of membrane integrity and leakage of cell contents and (b) a decrease in intracellular NAD^+ per viable cell when calculated based on NAD^+ in the cellular fraction alone. For severely damaged cells, correcting total NAD^+ levels extracted from cells plus medium for the SM-induced loss of viable cells may overestimate the intracellular levels of this coenzyme and underestimate the effect of SM.

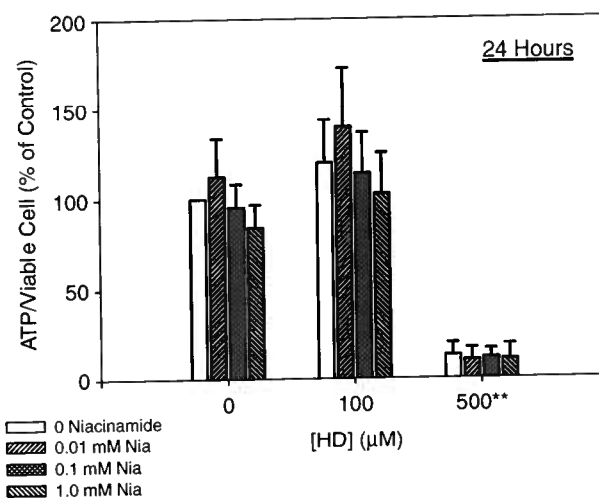


Figure 7 ATP contents of HEK corrected for SM-induced cell loss. Data are expressed as Mean \pm SEM ($n = 5$). **Significantly different from 0 and 100 μ M SM.

The inhibition of glucose metabolism that we observed both in this study and in our earlier work (12) is characterized by a shift in metabolism toward more lactate production than can be accounted for by metabolism of glucose alone, as evidenced by the differing levels of inhibition seen for these two end-points (Fig. 3). The cause of the increase in ATP content seen at 4 h after exposure to 100 μ M SM is not known. However, it may result from increased catabolic activity in response to the energy demands of repairing SM-induced damage to DNA and other macromolecules. This conclusion is supported by the results of our previous studies (12) in which about half of our experiments showed increased glycolytic activity in the first 8 h after exposure to 30–170 μ M SM. On the other hand, we cannot rule out the possibility that the increased ATP results from inhibition of energy-requiring processes less critical to cell survival (e.g., cell division), as an adaptive response to the initial metabolic insult.

In this study, 1 mM niacinamide (but not 0.01 or 0.1 mM) provided at least partial, and in some cases complete, protection against SM-induced NAD^+ depletion (Fig. 4), in agreement with results from other laboratories (3–5, 7–9). However, even under conditions in which NAD^+ levels were fully protected we observed no protection against the loss of cell viability, the decrease in total cell numbers, the inhibition of glucose metabolism or the depletion of ATP.

The inability of niacinamide to prevent the SM-induced inhibition of glucose metabolism (glucose utilization and lactate production) in cultured HEK even when NAD^+ levels were fully protected is in line with results of our earlier studies (12), which showed only a partial correlation between NAD^+ depletion and the inhibition of glycolysis. We concluded from those studies that the metabolic inhibition in HEK was not solely a function of NAD^+ depletion, but rather resulted from a combination of factors influenced by SM. That conclusion is supported by the current results, as well as by the similar findings of Mol et al. (4) for glucose

utilization by HEK. Interestingly, in later experiments on human skin cultures, Mol et al. (10) showed that niacinamide protected against both NAD^+ depletion and inhibition of glucose uptake, but did not protect against SM-induced histopathology, suggesting differences in the *ex vivo* system that may need to be examined further.

The results to date of studies on the ability of niacinamide to prevent SM-induced cell death have been conflicting. In the work reported here, niacinamide did not protect HEK from SM-induced cytotoxicity despite its preservation of intracellular NAD^+ stores. Similar results were seen by Lin et al. (15), who saw no correlation in rat keratinocytes between cytotoxicity and SM-induced NAD^+ depletion or its prevention by niacinamide. More recently, Blank et al. (9) also reported protection against NAD^+ depletion but only marginal protection against cytotoxicity in SM-exposed HEK. On the other hand, 1 mM niacinamide has been reported by Smith et al. (5) to provide partial protection of HEK against SM-induced losses of NAD^+ and cell viability, and by Meier et al. (8) and Smith et al. (3,5) to preserve NAD^+ and cell viability in peripheral blood lymphocytes (PBL).

The effects of SM and niacinamide on ATP reported herein are consistent with those reported by Mol and deVries-van de Ruit (7) in HEK (i.e., ATP depletion as a late event coincident with cell death; no protection by niacinamide). Hinshaw et al. (16) have also reported SM-induced loss of ATP in HEK as well as in bovine pulmonary endothelial cells. In the latter case, ATP loss was seen as early as 5 h after exposure, but only at very high concentrations of SM (500–1000 μM). In contrast, Meier et al. (8) have reported ATP depletion in PBL as early as 30 min after exposure to 300 μM SM and protection of ATP levels by 1 mM niacinamide.

Although the reasons for the discrepancies among the niacinamide and ATP studies are not entirely clear, it is evident that one contributory factor may be the cellular model in which the results were obtained. HEK and PBL are entirely different cell types with widely divergent physiological and biochemical characteristics. In addition, there are reports in the literature indicating that HEK respond quite differently to SM than do PBL. For example, Smith et al. (5) have observed that both resting and actively proliferating PBL are more sensitive than HEK to the cytotoxic effects of SM, and Moser and Meier (17) found qualitative differences in apoptotic markers between HEK and PBL upon exposure to SM alone or in the presence of PARP inhibitors. The latter study suggests the possibility that the mechanisms of SM-induced cell death may differ in the two cell types. In view of this it is perhaps not surprising to find that SM affects the energetics of HEK and PBL differently and that the protective ability of niacinamide also differs.

The differences in the responses of HEK and PBL to SM point out the importance of choosing the most appropriate *in vitro* cell model to use for the study of SM injury. PBL are easy to obtain in large numbers and, therefore, both suitable and preferred for screening studies requiring a large number of cells. We have chosen HEKs for our biochemical studies because they are derived from the target tissue of interest (basal epidermal cells of the skin). As such, they provide a better approximation of the *in vivo* system and a more relevant basis for the development of mechanism-based therapies.

In this article we have shown that in cultured HEK the protective effects of the PARP inhibitor niacinamide against SM-induced NAD^+ depletion do not extend to

protection against cytotoxicity, inhibition of glucose metabolism, or depletion of intracellular energy stores. In addition, the data support our original conclusion (12) that loss of NAD^+ is not the sole cause of the metabolic inhibition that results from exposure to SM and suggest the possibility that SM affects glycolysis directly. It is evident from our data that the effects of SM on cellular metabolism and the role of NAD^+ depletion in the cutaneous injury induced by SM are more complex than originally proposed (2). The simple cascade of SM-induced events (DNA alkylation \rightarrow PARP activation \rightarrow NAD^+ depletion \rightarrow glycolytic inhibition \rightarrow energy depletion \rightarrow cell death \rightarrow \rightarrow \rightarrow vesication) is no longer entirely valid and must be revised.

Although perhaps not the primary cause of SM-induced injury, loss of metabolic activity results in loss of energy required for vital cell functions that keep the cell alive and repair cell and tissue damage. Knowledge of the mechanisms of SM-induced metabolic injury can play a key role in the process of devising new therapeutic and/or prophylactic strategies to prevent cell death and promote optimal healing.

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